ARTICLES

Pathogenicity of porcine respiratory coronavirus isolated in Québec

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Abstract

Porcine respiratory coronavirus (PRCV) is present in many countries, including Canada, but controversy still exists concerning its pathogenicity.

Eight-week-old piglets were inoculated intratracheally with a Quebec PRCV isolate (1Q90). Two contact piglets were kept with the inoculated animals. Three animals served as control.

Polypnea and dyspnea were the main clinical signs observed. Diffuse bronchioloalveolar damage occurred 24 hours postinoculation. Changes compatible with bronchointerstitial pneumonia were present six days postinoculation. The inoculated virus was recovered from the respiratory tract and mesenteric lymph nodes, but not from the digestive tract, of the inoculated as well as the contact piglets. No virus was isolated from the control piglets.

The development of clinical signs and histopathological changes in inoculated as well as in contact piglets and the reisolation of the inoculated virus demonstrated that PRCV can be an important respiratory pathogen.

Résumé

Pathogénicité d'un coronavirus respiratoire porcin isolé au Québec

Le coronavirus respiratoire porcin (PRCV) est présent dans plusieurs pays, y compris le Canada, mais son rôle en tant qu'agent pathogène reste encore controversé.

Huit porcelets âgés d'une semaine ont été inoculés par voie intratrachéale par un isolat québécois de PRCV (1Q90). Deux autres porcelets ont été maintenus en contact avec les animaux infectés. Trois porcelets ont servi de contrôles.

De la polypnée et de la dyspnée sont apparues chez les animaux infectés et les porcelets de contact. Un dommage bronchiolo-alvéolaire diffus a été observé 24 heures post-inoculation (PI). Des lésions de pneumonie broncho-interstitielle étaient présentes 6 jours PI. Le virus inoculé a été réisolé à partir du système respiratoire et des ganglions mésentériques mais pas à partir du système digestif des porcelets infectés et des animaux de contact. L'isolement viral a été négatif pour les trois sujets contrôles.

L'apparition de signes cliniques et de changements histologiques compatibles avec une infection virale, ainsi que le réisolement du virus inoculé démontrent que le PRCV peut être un pathogène respiratoire significatif.

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Introduction

Transmissible gastroenteritis is a highly contagious enteric disease of swine (1,2) that is caused by a coronavirus called transmissible gastroenteritis virus (TGEV). Transmissible gastroenteritis virus was first isolated in 1946 (3). It is an enteropathogenic coronavirus that replicates in the absorptive cells of the villi in the small intestine and causes rapid and extensive loss of epithelial cells. This results in atrophy of villi and severe watery diarrhea. Outbreaks are characterized by high mortality, which approaches 100% in piglets under two weeks of age (4). The TGEV can infect the respiratory tract without causing pneumonia (5).

A new porcine respiratory coronavirus (PRCV), which has a close antigenic relationship to the TGEV, suddenly emerged in 1983 in Belgium (6). The porcine respiratory coronavirus has spread rapidly in most, if not all, European countries, where it now persists enzootically (6–12). Recently, PRCV has been reported to be present in the United States (13).

Infection with PRCV induces antibodies that neutralize classical enteric TGEV. Conventional serological tests do not distinguish between PRCV- and TGEV-infected animals. Despite the antigenic relatedness of PRCV and TGEV, they can be differentiated with monoclonal antibodies (MAbs) (11,14–16). Thus, some non-neutralizing MAbs are employed in blocking assays to distinguish serologically between TGEV and PRCV infection (11,15,17–19).

Pathogenetic studies have shown that PRCV replicates at a high titer in the respiratory tract, but to a very low extent in the gut (20,21). The significance of this virus as a pathogen is still unclear. Although initially considered nonpathogenic (6), PRCV has subsequently been linked with field outbreaks of respiratory disease (9). Respiratory disorders and pulmonary lesions have also been demonstrated following experimental infections (11,22).

The objective of our work was to study the pathogenic role of a Quebec isolate of PRCV, 1Q90, and to determine its distribution and effects in selected tissues by virus isolation, immunofluorescence, and histopathology.

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Materials and methods

Cell cultures

Continuous swine testicle (ST) cell lines were used for the propagation, isolation, and titration of virus and for the virus neutralization test. Cells were grown in 150 cm^2 tissue culture flasks (Corning Glass Works. Corning, New York, USA) in Iscove's modification of Dulbecco's medium (IMDM) (Gibco, Grand Island, New York, USA) supplemented with 10% fetal bovine serum (FBS) (Hyclone Laboratories Inc., Logan, Utah, USA) and gentamicin (50 μg/mL) (Sigma, St Louis, Missouri, USA). The maintenance medium contained 2% FBS.

Viral stock

The virus used in this study had been isolated for the first time in 1990 from the lungs of a three-week-old piglet showing dyspnea (23). This isolate was identified as PRCV with MAbs 1DB12 and 6AC3 (14) conjugated with peroxidase. The isolate was passaged three times in two- to three-week-old piglets by the intratracheal inoculation of the supernatant of lung homogenate, supplemented with gentamicin. After the third passage in piglets, the virus was passaged twice on ST cells and titrated by a micromethod (19). The infectious titer of the inoculum obtained was $10^{8.5}$ $TCID_{50}$/mL. This isolate was named PRCV 1Q90.

Experimental animals

A pregnant sow was selected from a minimal disease herd. Pigs in this herd had no antibodies against TGEV or PRCV and no history of gastroenteritis or respiratory disease. The nasal cavity and tonsil of this sow were swabbed and cultured for PRCV. The PRCV was not isolated. The competitive inhibition ELISA and virus neutralization test (19) did not detect antibodies against PRCV or TGEV in the sow's serum. The sow gave birth to 13 piglets. Before experimental infection with PRCV 1Q90, the sow and 10 of her piglets (piglets 1–10) were placed in an isolation room. Her other three piglets (piglets 11-13) were placed in another isolation room. These three piglets were fed milk (Enfalac, Mead Johnson Canada, Candiac, Quebec) three times a day.

Experimental design

Piglets were weighed individually and tranquillized by an intramuscular (IM) injection of 0.25 mL/4.5 kg body weight (BW) of azaperone (Stresnil, Janssen Pharmaceutica Inc., Mississauga, Ontario) at one week of age. Fifteen minutes later, they were given an IM injection of 20 mg/kg BW of ketamine (Rogarsetic, rogar/STB Inc., London, Ontario). They were then intubated with the aid of a laryngoscope while being held in left lateral recumbency. Eight (piglets 1–8) of the 10 piglets kept with the dam received by the intratracheal route $10^{8.5}$ $TCID_{50}$ of PRCV 1Q90 in 5 mL of IMDM, while piglets 9 and 10 were not inoculated. Piglets 11, 12, and 13 received 5 mL of only IMDM in the same manner.

Food consumption, rectal temperature, and clinical signs were noted daily. Each piglet was euthanized by the intracardiac injection of 0.3 mL/kg BW of T-61 (Hoechst Canada Inc., Montréal, Québec), weighed, and necropsied. Samples from tonsils, trachea, lungs (cranial, middle, accessory, and caudal lobes), bronchial and mesenteric lymph nodes, small intestine (duodenum; proximal, middle, and distal jejunum; ileum) were taken for virus isolation and immunofluorescent studies.

Viral isolation and titration

Twenty percent homogenates (wt/vol) were prepared in 0.01 M phosphate-buffered saline (PBS) (pH 7.4), using a mixer (Sorvall Omnimixer, Ivan Sorval Inc., Newton, Connecticut, USA), and clarified by centrifugation at 10,000 rpm for 30 min at 4°C. All samples for virus isolation and titration were inoculated onto confluent ST cell monolayers. For titration, 100 μL aliquots of serial, 10-fold dilutions of each sample preparation were added to eight wells of 96-well microtiter plates. After an adsorption period of one hour, the infected cell cultures were washed three times with PBS and reincubated at 37°C in IMDM containing 1% FBS and 300 μg/mL of gentamicin. Cultures were examined daily for five days for evidence of cytopathic effects. No second passage was made. Titers were expressed in $\log_{10}$ $TCID_{50}$/g of tissue.

Immunofluorescence

The samples collected for examination by immunofluorescence were mounted in gelatin capsules (Tissue Teck OCT Compound, Miles Inc., Elkhart, Indiana, USA) and frozen (Tissue Teck II Cryostat, Miles Inc.). Frozen sections (5μm–6 μm thick) were fixed in cold acetone for 10 min, then stained by direct immunofluorescence (20). A hyperimmune serum against TGEV, conjugated with fluorescein isothiocyanate, was used as conjugate (Institut Armand-Frappier, Laval, Québec) following standard procedures (20). The specificity of fluorescence was determined by the fluorescence inhibition test (20). A semiquantitative evaluation of immunofluorescence was made according to the number of fluorescing cells in each section (20) as follows:

$+ = < 5\%$ $++ = 6–25\%$ $+++ = 26–50\%$ $++++ = > 50\%$.

Histopathology

Spleen, kidneys, liver, brain, and samples of the tissues already taken for viral isolation and immunofluorescent studies were fixed in 10% neutral buffered formalin, embedded in paraffin, sectioned at 6 μm, and stained with hematoxylin, phloxine, and saffron.

Bacterial isolation

Lung samples were taken from each animal for bacterial isolation. Samples were cultured on blood agar and McConkey's agar (Difco, Detroit, Michigan, USA) at 37°C in 7.5% CO_2 for 48 h. Parallel samples were inoculated into mycoplasma N agar and broth (Difco) and cultured for seven to nine days. A semiquantitative evaluation was made as follows: − = no growth; + = slight growth; ++ = medium growth; +++ = stronger growth; ++++ = luxuriant growth.

Results

Clinical signs

The three piglets inoculated with only IMDM remained healthy, whereas piglets 2 to 7, infected with PRCV, showed signs of severe respiratory distress within 24 h,

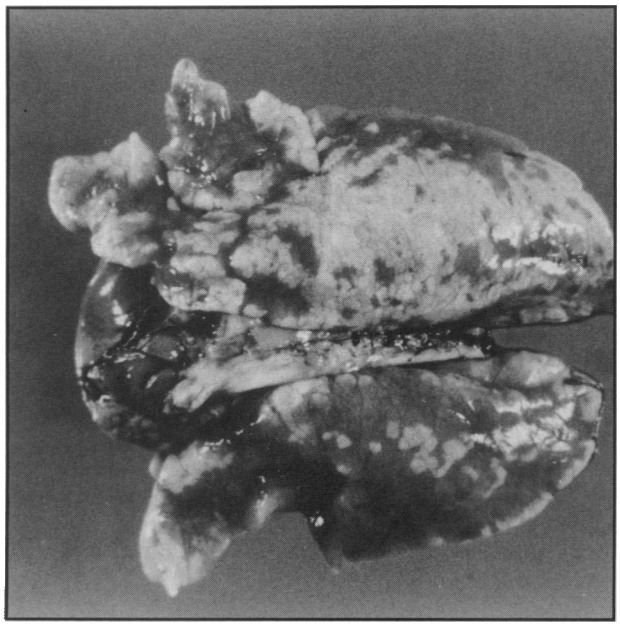

Figure 1. Macroscopic lesions of the lungs of piglet 3: the left lung was markedly consolidated four days postinoculation.

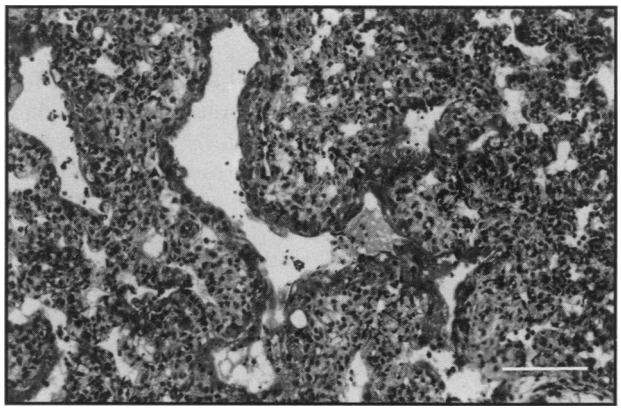

Figure 2. Necrosis of bronchiolar epithelium, with presence of fibrin, hyaline membranes, edema and necrotic cells in bronchiolar and alveolar lumina. Piglet 5; three days postinoculation (Hematoxylin, phloxine, and saffron stain). Bar = 50 μm.

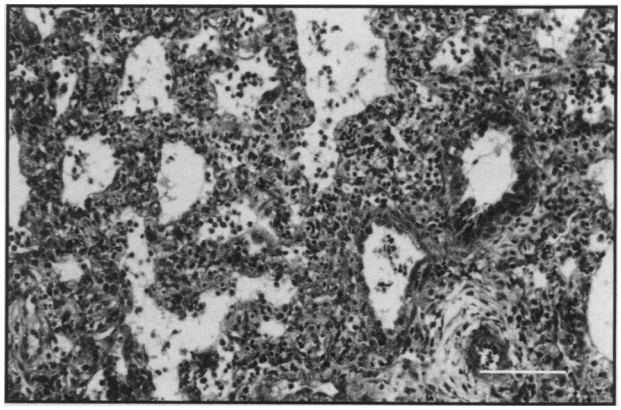

Figure 3. Regeneration of bronchiolar epithelium with thickening of alveolar septa and presence of necrotic cells in alveolar lumina. Piglet 3; four days postinoculation (Hematoxylin, phloxine, and saffron stain). Bar = 50 μm.

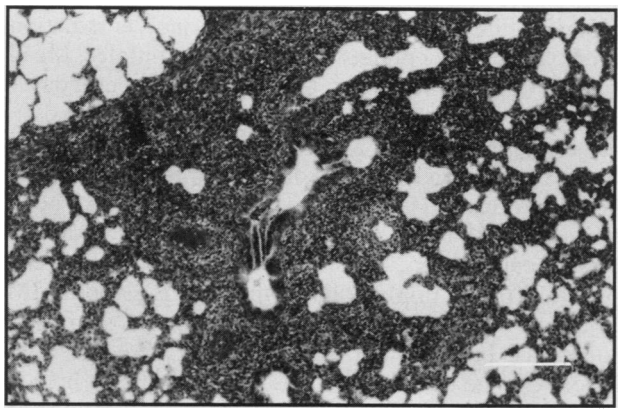

Figure 4. Bronchointerstitial pneumonia, with hyperplasia of peribronchiolar and perivascular lymph nodules, and severe thickening of alveolar septa. Piglet 1; seven days postinoculation (Hematoxylin, phloxine, and saffron stain). Bar = 200 μm.

including rapid breathing and dyspnea, and were euthanized in extremis. Depression and anorexia, accompanied by a significant loss of weight, were also observed in piglets 2 to 7, which proved to be more severely affected. In the terminal phase of the disease, these piglets looked extremely weak, were in lateral recumbency, and were hypothermic. Piglets 1 and 8, however, had only mild respiratory signs. The contact piglets 9 and 10 showed signs of respiratory distress on day 4 postinoculation, characterized by polypnea and hyperthermia (40.0°C–40.7°C), which lasted four to five days. The morbidity and the mortality rates reached 100% and 60%, respectively.

Gross lesions

Macroscopic lesions were limited to the lungs and tracheobronchial lymph nodes. In piglet 4, which died one day after inoculation, the lungs were markedly congested, emphysematous, and firm, and the bronchial lymph nodes were enlarged.

In piglets 2 and 6, which died two days after inoculation, the lungs were firm. Affected lobes were greyish-pink. A few superficial emphysematous bullae were also present. The left lung of piglet 6 was more affected than the right one. The bronchial lymph nodes of these two piglets appeared normal. Hydropericardium was present in both piglets.

In piglets 5, 3, and 7, which died on the days 3, 4, and 5 postinoculation, respectively, the left lung was completely firm and plum colored; the right lung also had some areas of marked consolidation (Figure 1). The bronchial lymph nodes were slightly enlarged. Hydropericardium was only observed in piglet 5.

In piglets 8 and 1, euthanized on days 6 and 7 postinoculation, respectively, lesions were less pronounced. The lungs of piglet 8 looked normal, but its bronchial lymph nodes were hemorrhagic and slightly enlarged. In piglet 1, a few small foci of consolidation were seen in both lungs; the bronchial lymph nodes, however, appeared normal.

In piglets 9 and 10, the pulmonary lobes had areas of consolidation of various sizes. The bronchial lymph nodes were hemorrhagic and slightly enlarged.

No lesions were present in the three control piglets, 11, 12, and 13.

Table 1. Isolations and titrations of porcine respiratory coronavirus from various tissues

	Inoculated animals								Contact		Controls		
Piglet No.	4	2	6	5	3	7	8	1	10	9	11	12	13
Days post-inoculation	1	2	2	3	4	5	6	7	8	9	1	4	7
Tonsils	3.3	5.3	3.6	4.8	3.7	0	0	0	0	0	0	0	0
Trachea	4.9	5.9	6.2	5.7	4.6	6.6	0	0	1.0	0	0	0	0
Lung: Cranial lobe	5.9	7.2	7.3	7.4	7.6	6.4	0	0	5.6	5.3	0	0	0
Accessory lobe	5.9	7.4	7.0	7.9	7.9	7.3	0	0	2.3	3.8	0	0	0
Middle lobe	6.1	7.6	8.1	6.8	7.7	6.7	0	3.7	5.3	5.6	0	0	0
Caudal lobe	7.6	7.6	7.6	7.2	7.2	6.8	0	4.1	4.8	5.3	0	0	0
Bronchial lymph nodes	5.8	5.4	5.1	4.2	4.9	3.1	0	0	0	0	0	0	0
Mesenteric lymph nodes	1.8	3.4	3.5	0	0	0	0	0	0	0	0	0	0
Duodenum	0	0	0	0	0	0	0	0	0	0	0	0	0
Jejunum	0	0	0	0	0	0	0	0	0	0	0	0	0
Ileum	0	0	0	0	0	0	0	0	0	0	0	0	0

Virus titer in log $^{10\text{-}1}$

Histological lesions

Significant histopathological changes were limited to the trachea, lungs, and spleen.

The bronchiolar epithelium was absent or necrotic 24 h postinoculation (piglet 4). Fibrin and hyaline membranes were mixed with macrophages, occasional neutrophils, and necrotic cells in alveolar lumina. Alveolar septa were thickened by intense congestion and focal hyperplasia of type II pneumocytes. Splenic follicles were severely depleted.

Similar pulmonary and splenic changes were observed in piglets 2 and 6, euthanized two days postinoculation. Mild infiltration of alveolar septa by mononuclear cells of histiocytic type and some regeneration of bronchiolar epithelium were noted in piglet 6. Focal to complete loss of tracheal epithelial cilia, without associated inflammation, was observed in those two piglets.

Piglet 5 (euthanized three days postinoculation) showed similar changes to those of the preceeding piglets. However, the infiltration of alveolar septa by mononuclear cells and the regeneration of bronchiolar epithelium were more severe (Figure 2).

Hyperplasia of peribronchiolar and perivascular lymph nodules and of type II pneumocytes was noted four days postinoculation (piglet 3). The pulmonary (Figure 3) and splenic changes were of the same type as those of piglet 5. Similar pulmonary observations were made five days postinoculation (piglet 7). No extrapulmonary lesions were noted in that piglet.

Lymphocytes and some neutrophils had infiltrated the tracheal epithelium and superficial parts of the lamina propria of piglet 8 (six days postinoculation). Multifocal, confluent, interstitial pneumonia with infiltration of the alveolar septa by mononuclear cells was present. Hyperplasia of type II pneumocytes and of peribronchiolar and perivascular lymph nodules was observed. Macrophages and, occasionally, fibrin were observed in alveolar lumina. Necrosis and regeneration of bronchiolar epithelium were observed. No extrapulmonary lesions were seen.

Similar but less intense pulmonary lesions with the presence of necrotic cells in bronchiolar and alveolar lumina were observed in piglet 1, euthanized seven days postinoculation (Figure 4). Severe lymphoid depletion of the spleen was also noted.

The contact animals (piglets 9 and 10) developed similar lesions to those of inoculated piglets, without tracheal (piglet 10) or splenic changes (piglet 9).

No significant microscopical changes were observed in the tissues of control animals (piglets 11, 12, and 13).

Viral isolation

Inoculation of the PRCV isolate onto the ST cultures induced the formation of typical syncytia between one

Table 2. Detection of porcine respiratory coronavirus from various tissues by immunofluorescence

	Inoculated animals								Contact		Controls		
Piglet No.	4	2	6	5	3	7	8	1	10	9	11	12	13
Days post inoculation	1	2	2	3	4	5	6	7	8	9	1	4	7
Tonsils	−	+	−	−	−	−	−	−	−	−	−	−	−
Trachea		+	++	+	−	++	−	−	−	−	−	−	−
Lung: Cranial lobe	++++	+++	+++	+++	++++	++	−	−	++	+	−	−	−
Accessory lobe	++++	+++	+++	+++	+++	+++	−	−	−	−	−	−	−
Middlel lobe	+++	+++	+++	+++	++++	++	−	−	+	++	−	−	−
Caudal lobe	+++++	+++	++++	++++	++++	++	−	−	+	+	−	−	−
Bronchial lymph nodes	++	+	+	−	+	−	−	−	−	−	−	−	−
Mesenteric lymph nodes	−	−	−	−	−	−	−	−	−	−	−	−	−
Duodenum	−	−	−	−	−	−	−	−	−	−	−	−	−
Jejunum	−	−	−	−	−	−	−	−	−	−	−	−	−
Ileum	−	−	−	−	−	−	−	−	−	−	−	−	−

A semi-quantitative evaluation of immunofluorescence was made according to the number of fluorescing cells in each section:
− = none + = < 5% ++ = 6–25% +++ = 26–50% ++++ = > 50%

and three days after inoculation. The results of the viral isolations and titrations of samples from experimentally-infected pigs are presented in Table 1. Virus was isolated from tonsils, trachea, lungs, and bronchial lymph nodes. It was isolated inconsistently from mesenteric lymph nodes. The highest titers were detected in lungs (up to 10^8 $TCID_{50}$/g of lung tissue). Virus was not isolated from the digestive tract.

Immunofluorescence

Results of the immunofluorescent study are summarized in Table 2. Viral antigens in the respiratory tract were detected in the cytoplasm of epithelial cells of trachea and lung tissue. Fluorescence in lungs was seen in epithelial cells of alveoli, bronchioles, and bronchi. The percentage of fluorescent cells in those tissues was about the same from one to five days postinoculation. At this time, generally more than 50% of the alveolar cells were fluorescing. Viral antigens were present in the cytoplasm of a few superficial tonsillar epithelial cells in one piglet. Viral antigens could be detected in a few cells in bronchial lymph nodes but not in mesenteric lymph nodes. Immunofluorescence was not detected in the digestive tract.

Bacterial isolation

The results of bacteriological studies are given in Table 3. A few nonpathogenic microorganisms were cultured from the lungs of five piglets.

Discussion

The pathogenic role of isolate PRCV 1Q90 was suspected when it was first isolated (23) from a piglet with a pneumonia similar to that already described (9,11,22). The clinical signs observed in our experimental piglets were more severe than any previously reported. After successive passages in piglets, inoculum doses less than 10^5–10^6 $TCID_{50}$ had induced respiratory signs and the formation of macroscopic and microscopic pulmonary lesions (23). Therefore, an inoculation dose of $10^{8.5}$ $TCID_{50}$ was chosen to obtain a more pronounced effect. Commercial piglets were deliberately chosen to simulate field conditions. Furthermore, commercial piglets have been used by other workers (13). Likewise, one-week-old piglets (and younger) have also been used previously (13,20,21).

Oronasal, intranasal, and intratracheal routes, as well as aerosols, have been used by other workers (13,20,21,22). The inoculum used in previous studies var-

Table 3. Isolation of bacteria from lungs

Piglet No.	2	3	4	5	6[a]
Streptococcus suis					
— serotype 12			+		
— serotype 21	+++				+++
Escherichia coli	+++	+	+		+++
Klebsiella pneumoniae					+
Staphylococcus nonhemolytic				+	++
Streptococcus nonhemolytic		+			

[a]Piglets 1, 7–13: negative

ied between $10^{3.4}$ and $10^{8.24}$ $TCID_{50}$. Oronasal administration of the latter dose was too low to induce disease (13). However, by using the intratracheal route and older piglets, some workers induced severe disease, sometimes accompanied by a low mortality rate (11, 22). Such results would indicate that the age of piglets, inoculation route, and dose of inoculum represent important factors in respiratory infections caused by PRCV. Such observations suggest the existence of pathogenic and nonpathogenic viral strains. For instance, most of the Quebec swine herds tested in the course of our study (19) proved to be seropositive to PRCV, although none had exhibited respiratory symptoms.

We observed significant macroscopic lesions in the lungs and, occasionally, the tracheobronchial lymph nodes. Similar lesions had already been observed after experimental infection with PRCV in both the presence and the absence of clinical signs (11,21,22). Microscopical changes in our experiment were limited to the trachea, lungs, and spleen. The first changes noted in the lungs (fibrin, hyaline membranes, necrotic cells, some erythrocytes in alveoli, and necrosis of bronchiolar epithelium) are compatible with diffuse bronchioloalveolar damage. The bronchointerstitial pneumonia noted six days postinoculation has already been observed after experimental infection with PRCV (20,21,24). The variation in the intensity of lesions may reflect some difference in the pathogenicity of the viral strain and/or viral dose used.

Tracheal lesions (piglets 1,2,5–8) were characterized by partial loss of cilia from tracheal epithelium and mild multifocal lymphocytic tracheitis, as has been noted by other workers (21). The severe lymphoid depletion seen in the spleen of our piglets has never been reported following experimental infection by PRCV. It may reflect a possible viremia and/or severe stress subsequent to respiratory infection.

The virus was recovered from tonsils, trachea, lungs, and bronchial and mesenteric lymph nodes. The fact that the virus could be recovered from mesenteric lymph nodes indicates that there was a viremia (20). The results of immunofluorescent studies were almost identical to those of viral recovery, and the distribution of viral antigen was found to be similar to that already reported (20).

The results of previous studies indicating that the tissues of the respiratory tract are the primary site for the replication of PRCV in swine have been confirmed in our study (6,20,21). However, we did not succeed in demonstrating the presence of the virus in the digestive tract, except in the mesenteric lymph nodes, although European workers have reported isolating it from the digestive tract (20,21).

Contact piglets 9 and 10 exhibited respiratory symptoms and hyperthermia, and the virus was isolated from both of them. Macroscopic and microscopic lesions were similar to those found in experimentally inoculated piglets. These findings clearly indicate that the virus can be transmitted through aerosols, as has already been reported (13,25). The manifestation of respiratory signs by contact piglets confirms that PRCV 1Q90 is a respiratory pathogen and is contagious.

No other pathogenic agent could be isolated from the lungs of the inoculated, contact, or control piglets. The bacteria isolated from the lungs of these piglets have not been incriminated in porcine respiratory problems in Quebec (27,28,29).

We conclude that our clinical, pathological, and virological findings confirm a pathogenic role for PRCV isolate 1Q90. A close correlation has been noted between the severity of respiratory symptoms, the extent of macroscopic and microscopic lesions, the recovery of the virus, and the results of immunofluorescence.

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